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AT

(54) Title: LIPOSOME DELIVERY METHOD FOR DECREASING THE TOXICITY OF AN ANTITUMOR DRUG

(57) Abstract

A method for decreasing the toxicity of an antitumor drug. The drug is entrapped in liposomes also containing a drug-protective compound.

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Liposome Delivery Method for Decreasing the
Toxicity of an Antitumor Drug

Background and Summary

The following references are referred to by
corresponding number in this application:

- 5 1. Forssen, E. A. and Tokes, Z. A. In vitro and in vivo studies with adriamycin liposomes. Biochem Biophys Res Comm 91:1295-1301 (1979).
2. Forssen, E. A. and Tokes, Z. A. Use of anionic liposomes for the reduction of chronic doxorubicin
10 induced cardiotoxicity. Proc Nat Acad Sci USA 78:1873-1877 (1981).
3. Maslow, D. E., Mayhew, E., Olson, F., and Rustum, Y. Reduction of the inhibitory effect of Adriamycin on myocardial contraction in vitro by entrapment in liposomes. Proc Am Assoc Cancer Res 21:281 (1980).
15 4. Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka, F. Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. Eur J Cancer Clin Oncol 18(2):167(1982).
- 20 5. Rahman, A., Kessler, A., More, N., Sikie, B., Rowden, G., Woolley, P. and Schein, P. S. Liposomal protection of adriamycin-induced cardiotoxicity in mice. Cancer Res 40:1532-1537 (1980).
- 25 6. Diplock, A. T., Lucy, J. A., Verrinder, M. and Zieliniewski, A. Alpha-tocopherol and the



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- permeability to glucose and chromate of unsaturated liposomes. Febs Lett 82:341-344 (1977).
7. Fukazawa, K., Ikeno, H., Tokumura, A., and Tsukatani, H. Effect of alpha-tocopherol incorporation on glucose permeability and phase transition of lecithin liposomes. Chem Phys Lip 23:13-22 (1979).
8. Myers, E., McGuire, W. and Young, R., Adriamycin: amelioration of toxicity by alpha-tocopherol. Cancer Treatment Reports 60:961-962 (1976).
9. Sonneveld, P. Effect of alpha-tocopherol on the cardiotoxicity of adriamycin in the rat. Cancer Treatment Reports 62:1033-1036 (1978).
10. Wang, Y.-M., Madanat, F. F., Kimball, J. C., Gleiser, C. A., Ali, M. K., Kaufman, M. Q. and van Eyes, J. Effect of vitamin E against Adriamycin-induced toxicity in rabbits. Cancer Res 49:1022-1027 (1980).
11. Mayhew, E. and Rustum, Y. M. Effects of liposome entrapped adriamycin (AM) against ovarian tumor M5076 "metastatic" to the liver. Proc Am Assoc Cancer Res 23:170 (Abstract #668) (March, 1982).
12. Hunt, C. A. and Tsang, S. Alpha-tocopherol retards auto-oxidation and prolongs the shelf life of liposomes. Int J Pharmaceutics 8:101-110 (1981).
13. Konigs, A. W. T., Darren, J. and Trieling, W. B. Protection of liposomal lipids against radiation induced oxidative damage. Int J Radiat Biol 35:343-350 (1979).
14. Mayhew, E., Rustum, Y. M., Szoka, F. and Papahadjopoulos, D. Role of cholesterol in enhancing the anti-tumor activity of



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- 1-alpha-D-Arabinofuranosylcytosine entrapped in reverse phase evaporation vesicles. Cancer Treatment Reports 63:1923-1928 (1979).
15. Doroshaw, J. H., et al, J Clin Invest 68:1053 (1981).
- 5 16. Scheulen, M. E., et al, Proc Am Assoc Cancer Res 23:992(1983).
17. Rogan, A. M., et al, Science 224:994 (1984).
18. Juhl, H., et al, Biochem Biophys Res Commun 106(1):210 (1982).
- 10 19. Weiss, B., Annals N Y Acad Sci 356:319 (1980).

Entrapment of adriamycin (AM) in lipid bilayer vesicles, or liposomes has been reported to reduce the toxicity of this drug in animals, probably by

15 cardiotoxicity reduction (references 1-5). In addition, alpha-tocopherol (vitamin E), which has been reported to stabilize phospholipid membranes (references 6,7), has also been reported to reduce the cardiotoxicity of AM in animals when both compounds are administered in the free

20 form (references 8-10). A study reported by two of the inventors herein suggests that AM entrapped in liposomes containing entrapped alpha-tocopherol is more effective against tumor growth in mice than is free AM (reference 11). Studies conducted in support of the parent

25 application and reported herein, indicate that at the AM dosage where increased effectiveness of liposome-entrapped AM is observed, the increase is about the same whether or not the entrapping liposomes contain alpha-tocopherol. None of the above-mentioned studies

30 suggest that liposomes containing both entrapped AM and



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alpha-tocopherol have important therapeutic properties not found in liposomes containing entrapped AM only.

An important feature of the invention, therefore, is the finding that liposomes containing both AM and alpha-tocopherol are substantially less toxic than other AM formulations whose toxicity has been studied. These findings have led to an improved method for decreasing the toxicity of drugs generally. The method comprises trapping the drug and a drug-protective compound, at a selected ratio, in the same lipid bilayer vesicles. The drug-protective compound is one which itself decreases the toxicity of the drug when both compounds are administered in free form. In a specific embodiment of the invention, the anti-tumor drug is AM, the drug-protective compound is alpha-tocopherol and the toxicity of AM when encapsulated in liposomes containing alpha-tocopherol, is decreased more than about 60% over that of AM entrapped in vesicles containing no alpha-tocopherol.

It is one object of the invention, therefore, to provide a novel method for reducing the toxicity of drugs.

A more particular object of the invention is to provide a method for reducing the toxicity of an anti-tumor drug, such as AM, by including the drug in liposomes also containing a drug-protective compound, such as alpha-tocopherol.

Still another object of the invention is to provide such a method which is applicable to a wide range of drugs and drug-protective compounds, both soluble and lipophilic.



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A further object of the invention is to provide a therapeutic agent comprising liposomes containing an entrapped anti-tumor drug, such as AM, and a coentrapped drug-protective compound.

- 5 These and other objects and features of the invention will become more fully apparent from the following detailed description of the invention.

Detailed Description of the Invention

Phospholipids and purified cholesterol were
10 prepared as described previously (reference 13).
Multilamellar liposomes were made by first mixing 60
µmoles of a 1:4:4 molar ratio mixture of
phosphatidylglycerol; phosphatidylcholine and cholesterol
(in chloroform) with 1.5 mg AM (in methanol) and 0.6
15 µmoles alpha-tocopherol (in chloroform). AM
(doxorubicin) was obtained from Adria Corp. (Columbus,
OH). The AM-containing lipid mixture was evaporated by
a rotary evaporation at room temperature. Phosphate
buffered saline free of calcium and magnesium (PBS), pH
20 7.4 was added (1 ml/60 µmoles lipid) at 37°C and the
suspension was shaken at 37°C overnight. The
heterogeneous multilamellar liposome suspension which
formed was extruded through a 0.4 micron nucleopore
filter under 40-80 psi nitrogen pressure at room
25 temperature and centrifuged at 130,000 x g for 1 hr at
20°C to concentrate the liposomes and to remove much of
the non-entrapped AM. The liposomes were extensively
dialyzed against 100-200 volumes of PBS with stirring at
37°C. The percent of AM entrapped, determined by
30 fluorescence spectrophotometry, was between about 65%
and 70%. The calculated molar ratio of alpha-tocopherol



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to total lipids in the liposomes is about 1:100. Substantially all of the alpha-tocopherol was trapped in the liposomes. The same procedure was used to prepare liposomes entrapping AM but containing no
 5 alpha-tocopherol (by omitting alpha-tocopherol from the preparation) as described previously (4).

The therapeutic effects of AM were tested using DBA 2J mice injected intraperitoneally with 10^6 L1210 leukemia cells. The animals were treated one day later
 10 by intravenous injection of AM, either in the form of free AM, liposomes entrapping AM only (AM/liposomes), or liposomes entrapping both AM and alpha-tocopherol (AM-aT/liposomes). The dosages of AM administered, expressed in milligrams AM per kilogram animal body
 15 weight, are given at the left in Table I below. The day of death of the animals was recorded, and the mean survival time of each group was calculated. Each group contained from between 6 and 10 mice. The mean survival time and calculated standard deviations are shown at the
 20 three columns at the right in Table I.

Table I

dose AM (mg/kg)		Mean Survival Time (days) \pm S.D.		
		free AM	AM/liposomes	AM-aT/liposomes
25	0	7.2 \pm 0.4	-	-
	10	18.4 \pm 1.8	16.8 \pm 2.4	17.6 \pm 3.0
	20	12.2 \pm 1.6	17.6 \pm 2.0	16.8 \pm 1.6
	50	-	13.0 \pm 4.3	17.0 \pm 4.0

The data in Table I show that at a dosage of 10
 30 mg/kg, the anti-leukemic effectiveness of free AM was



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maintained, but not improved, by entrapment in liposomes alone or liposomes containing alpha-tocopherol. At the 20 mg/kg dosage level, the effectiveness of liposome-entrapped AM, either in the presence or absence of alpha-tocopherol was better than that of free AM at the same concentration, similar to what was reported in reference 11. However, at no AM dosage level was the effectiveness of AM entrapped in liposomes, either in the presence or absence of alpha-tocopherol, greater than the optimal, 10 mg/kg dosage level of free AM.

Similar studies were performed to determine the effect of the various AM formulations on long-term survival in mice which had been injected intraperitoneally with 10^5 L1210 leukemia cells. The infected animals were treated with intraperitoneal injections of AM (10 mg/kg body weight) administered either as free AM, AM encapsulated in liposomes, AM encapsulated in liposomes also containing alpha-tocopherol, or a mixed population of liposomes containing AM only and liposomes containing alpha-tocopherol only. Significantly, the only AM formulation that gave long-term survival (2 of 6 mice survived to 172 days after initial infection) was the liposome formulation containing coentrapped AM and alpha-tocopherol.

At least under certain therapeutic conditions, then, supplying the drug and drug-protective compound in the same liposome population produced therapeutic results which are superior to those obtained by administering the drug and drug protective compound in separate liposome populations. The results suggest that release of a drug (in this case, an anthracycline



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anti-tumor drug) and a drug-related compound (in this case, a drug protective compound) in the same localized liposome-target region may have important therapeutic consequences not realized heretofore.

- 5 The toxicity of the three AM preparations described with respect to Table I was tested against healthy DBA 2J mice. Groups of mice (6-10 mice/group) were injected with a single iv dose of free AM, liposomes entrapping AM only (AM/liposomes), and
- 10 liposomes entrapping both AM and alpha-tocopherol (AM-aT/liposomes) in doses of 2, 5, 10, 15, 20, 25, 30, 50, 75, 100 mg AM per kg animal body weight.

 In the experiment, which is reported in Table II below, it was found that the mice died at two

15 distinct time periods after administration: within 3-14 days, and between approximately 8-12 weeks after drug administration. The data are expressed in terms of LD_{50} , i.e., the dosage (in mg drug per kilogram of animal body weight) which produces death in half the

20 animals receiving the drug. The upper row in Table II gives the LD_{50} data for mice dying within 14 days after drug administration (acute), the lower row, for mice dying between 50 and 120 days after drug administration (chronic). The number of mice available

25 for determination of chronic toxicity (survival of acute toxicity) varied from between about 2 and 10 mice per group.



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Table II

	Mean LD ₅₀ (mg/kg) \pm S.D.		
	free AM	AM/liposomes	AM-aT/liposomes
acute	20 \pm 5	45 \pm 5	>75
5 chronic	12 \pm 5	30 \pm 5	50

The data in Table II confirm that both acute and chronic toxicity of AM are reduced more than 2-fold by encapsulating the drug in liposomes containing no alpha-tocopherol, as has been reported previously.

- 10 According to an important finding of the present invention, entrapping the drug in liposomes which also contain entrapped alpha-tocopherol further reduces acute and chronic toxicity more than about 60% with respect to liposomes entrapping AM alone. It is noted that 75
15 mg/kg is about the highest drug dosage which could be administered with a single intravenous injection. Therefore the LD₅₀ value for acute toxicity from AM-aT/liposomes may be somewhat higher than shown.

- The results presented herein suggest that it
20 may be possible to use a total cumulative dose of AM, after co-entrapment with alpha-tocopherol in liposomes, several times greater than that possible with the free drug. Alternatively, similar doses of the liposome-entrapped drug only could be used with reduced
25 risk of cardiotoxicity.

- Further reduction in drug toxicity may be achieved by increasing the relative amount of drug protective compound in the drug-containing liposomes. As noted above, drug-protective liposomes described
30 herein had an alpha-tocopherol to total lipid ratio of about 1:100. Using an alpha-tocopherol succinate, the



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ratio of alpha-tocopherol to total liposome lipids can be made much greater, preferably in the molar ratio range of 1:20 to 1:5, i.e., between about 5 and 20 mole percent alpha-tocopherol.

5 A preferred therapeutic agent of the invention comprises liposomes containing phospholipid, cholesterol, alpha-tocopherol succinate and AM at molar percentages of between about 30% and 70%, 20% and 50%, 5% and 20% and 0.2% and 15%, respectively.

10 The data supports the concept of using liposomes to carry more than one agent simultaneously, where one of the agents is a drug and the other agent is either a drug-protective compound, such as disclosed herein, or a drug-potentiating compound which promotes
15 the action of the drug at the site of drug delivery.

Examples of other drug-protective compounds, which have been shown to reduce anthracycline cardiac toxicity when administered in free form, include hydroxybutylated toluene, N-acetylcysteine (reference
20 15) and niacin and isocitrate (reference 16). In practicing the method of the present invention, these compounds would be coentrapped, for example, at encapsulated concentrations of between about 5-100 mg/ml in anthracycline-containing liposomes, to produce an
25 enhanced reduction in drug toxicity.

Compounds that potentiate anthracycline activity include agents that block calcium uptake, such as verapamil (reference 17), compounds that interfere with calcium mobilization from an intracellular store,
30 such as 8-(N,N-diethylamino)-octyl-3, 4,5-trimethoxybenzoate (TMB-8) (reference 18), or compounds that interfere with calcium binding to the protein



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calmodulin, such as trifluoroperazine, thioridazine, and other compounds noted in reference 19. These compounds would be included, at normal therapeutically effective doses, and/or maximum concentrations consistent with
5 liposome membrane stability, in liposomes also formulated to contain encapsulated an anthracycline drug, and administered in a suitable manner, such as is detailed above.

While the present invention has been
10 illustrated with respect to one particular embodiment, it will be appreciated by those skilled in the art that various changes and modifications can be made without departing from the scope and spirit of the invention.



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WHAT IS CLAIMED IS:

1. A method for decreasing the toxicity of a drug substantially below that produced when the drug alone is entrapped in lipid bilayer vesicles, or that produced when the drug and a drug-protective compound
5 are both administered in free form, the drug-protective compound being one which itself decreases the toxicity of the drug when administered with the drug in free form, said method comprising entrapping the drug and the compound, at a selected drug-to-compound ratio, in the
10 same lipid bilayer vesicles.
2. The method of claim 1, wherein the drug-protective compound includes an anthracycline drug.
3. The method of claim 2, wherein the drug protective compound is selected from the group
15 consisting of alpha-tocopherol, alpha-tocopherol succinate, N-acetylcysteine, niacin, isocitrate, and beta-hydroxybutylated toluene.
4. The method of claim 2, wherein the drug includes adriamycin, the drug-protective compound
20 includes alpha-tocopherol, and the toxicity of the drug entrapped in lipid vesicles also containing alpha-tocopherol, as measured in mice, is decreased at least about 50% below the toxicity of the drug entrapped in vesicles containing no alpha-tocopherol.
- 25 5. A therapeutic agent comprising liposomes containing phospholipid, cholesterol, alpha-tocopherol



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succinate, and adriamycin at molar percentages between about 30% and 70%, 20% and 50%, 5% and 20% and 0.2% and 15%, respectively.



INTERNATIONAL SEARCH REPORT

PCT/US84/1431

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁵

According to International Patent Classification (IPC) or to both National Classification and IPC ⁵

A61K 9/22, 9/52, 9/42, 31/685, 47/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System

Classification Symbols

U. S.

424/10, 38, 199, 365

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X, Y	US, A, 3,993,754, published 23 November 1976 Rahman et al.	1-5
X, Y	US, A, 4,256,632, published 17 March 1981 Levin et al.	1-5
X, Y	US, A, 4,263,428, published 21 April 1981 Apple et al.	1-5
X, Y, P	US, A, 4,419,348, published 06 December 1983 Rahman et al.	1-5
X, Y	US, A, 4,241,046, published 20 December 1980 Papahadjopoulos et al.	1-5
X, Y	N, Forssen, E. A. and Tokes, Z. A. <u>In vitro</u> and <u>in vivo</u> studies with adriamycin liposomes. <u>Biochem. Biophys. Res. Comm.</u> 91:1295-1301 (1979)	1-5

* Special categories of cited documents: ¹⁶

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search ³

20 November 1984

Date of Mailing of this International Search Report ³

Cue 26 NOV 1984

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ¹⁹

Ciro J. Faraci

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,Y	N, Forssen E. A. and Tokes, Z. A. Use of anionic liposomes for the reduction of chronic doxorubicin induced cardiotoxicity. <u>Proc. Nat. Acad. Sci. USA</u> 78:1873-1877 (1981)	1-5
X,Y	N, Maslow, D. E., Mayhew, E., Olson, F., and Rustum, Y. Reduction of the inhibitory effect of Adriamycin on myocardial contraction <u>in vitro</u> by entrapment in liposomes. <u>Proc. Am. Assoc. Cancer Res.</u> 21:281 (1980)	1-5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X,Y	N, Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka, F. Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. <u>Eur. J. Cancer</u> (in press)	1-5
X,Y	N, Rahman, A., Kessler, A., More, N., Sikie, B., Rowden, G., Woolley, P. and Schein, P.S. Liposomal protection of adriamycin-induced cardiotoxicity in mice. <u>Cancer Res.</u> 40: 1532-1537 (1980)	1-5
X,Y	N, Diplock, A.T., Lucy, J.A., Verrinder, M. and Zieliniewski, A. Alpha-tocopherol and the permeability to glucose and chromate of unsaturated liposomes. <u>Febs. Lett.</u>	1-5
X,Y	N, Fukazawa, K., Ikeno, H., Tokumura, A., and Tsukatani, H. Effect of alpha-tocopherol incorporation on glucose permeability and phase transition of lecithin liposomes. <u>Chem. Phys. Lip.</u> 23:13-22 (1979)	1-5
X,Y	N, Myers, E., McGuire, W. and Young, R. Adriamycin amelioration of toxicity by alpha-tocopherol. <u>Cancer Treatment Reports</u> 60:961-962 (1976)	1-5
X,Y	N, Sonneveld, P. Effect of alpha-tocopherol on the cardiotoxicity of adriamycin in the rat. <u>Cancer Treatment Reports</u> 62:1033-1036 (1978)	1-5
X,Y	N, Wang, Y.M., Madanat, F.F., Kimball, J.C., Gleiser, C.A., Ali, M.K., Kaufman, M.Q. and van Eyes, J. Effect of vitamin E against Adriamycin-induced toxicity in rabbits. <u>Cancer Res.</u> 49:1022-1027 (1980)	1-5
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